

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) Publication number:

0 307 434 B1

(12)

EUROPEAN PATENT SPECIFICATION(45) Date of publication of patent specification: **08.09.93** (51) Int. Cl.⁵: **C07K 15/00**(21) Application number: **88902525.0**(22) Date of filing: **18.03.88**(86) International application number:
PCT/GB88/00211(87) International publication number:
WO 88/07089 (22.09.88 88/21)(54) **ALTERED ANTIBODIES.**(30) Priority: **18.03.87 GB 8706425**
10.08.87 GB 8718897
01.12.87 GB 8728042(43) Date of publication of application:
22.03.89 Bulletin 89/12(45) Publication of the grant of the patent:
08.09.93 Bulletin 93/36(84) Designated Contracting States:
AT BE CH DE FR IT LI LU NL SE

(56) References cited:

Immunochemistry, volume 11, 1974, Pergamon Press (GB) R. Allan et al. "Studies on the complement-binding site of rabbit immunoglobulin G-I. Modification of tryptophan residues and their role in anticomplementary activity of rabbit IgG", pages 175-180

(73) Proprietor: **MEDICAL RESEARCH COUNCIL**
20 Park Crescent
London W1N 4AL(GB)(72) Inventor: **WINTER, Gregory, Paul**
64 Cavendish Avenue
Cambridge(GB)
Inventor: **DUNCAN, Alexander, Robert**
5 Harvey Road
Cambridge(GB)
Inventor: **BURTON, Dennis, Raymond**
41 Carsick Hill Road
Sheffield S10(GB)(74) Representative: **Matthews, Heather Clare et al**
Keith W Nash & Co Pearl Assurance House
90-92 Regent Street
Cambridge CB2 1DP (GB)

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid (Art. 99(1) European patent convention).

EP 0 307 434 B1

Chemical Abstracts, volume 99, no. 11, 12
September 1983, (Columbus, Ohio, US), T.
Hofstaetter et al.: "S-sulfonation: a reversible
chemical chemical modification of human
immunoglobulins permitting intravenous ap-
plication. II. Effect on Fc-mediated effector
functions", see page 423, abstract 86345v, &
Vox Sang. 1983, 45(2), 155-65

Nature, volume 332, 7 April 1988, A.R. Dun-
can et al.: "Localization of the binding site
for the human high-affinity Fc receptor on
IgG", pages 563-564

Chemical Abstracts, volume 93, no. 7, 18
August 1980, (Columbus, Ohio, US), F.
Vivanco-Martinez et al., page 703.

DescriptionField of invention

5 This invention relates to altered antibodies and concerns an antibody with an altered effector function, a method of producing such an antibody, and a process for altering an effector function of an antibody.

Background to the invention

10 Antibodies, or immunoglobulins, comprise two heavy chains linked together by disulphide bonds and two light chains, each light chain being linked to a respective heavy chain by disulphide bonds. The general structure of an antibody of class IgG (ie an immunoglobulin (Ig) of class gamma (G)) is shown schematically in Figure 1 of the accompanying drawings.

Each heavy chain has at one end a variable domain followed by a number of constant domains. Each light chain has a variable domain at one end and a constant domain at its other end, the light chain variable domain being aligned with the variable domain of the heavy chain and the light chain constant domain being aligned with the first constant domain of the heavy chain.

Antigen binds to antibodies via an antigen binding site in the variable domains of each pair of light and heavy chains. Other molecules, known as effector molecules, bind to other sites in the remainder of the molecule, ie other than the antigen binding sites, and this portion of antibody will be referred to herein as "the constant portion" of an antibody, such sites being located particularly in the Fc region constituted by the portions of the heavy chains extending beyond the ends of the light chains.

Antibodies have several effector functions mediated by binding of effector molecules. For example, binding of the C1 component of complement to antibodies activates the complement system. Activation of complement is important in the opsonisation and lysis of cell pathogens. The activation of complement also stimulates the inflammatory response and may also be involved in autoimmune hypersensitivity. Further, antibodies bind to cells via the Fc region, with a Fc receptor site on the antibody Fc region binding to a Fc receptor (FcR) on a cell. There are a number of Fc receptors which are specific for different classes of antibody, including IgG (gamma receptors), IgE (eta receptors), IgA (alpha receptors) and IgM (mu receptors). Binding of antibody to Fc receptors on cell surfaces triggers a number of important and diverse biological responses including engulfment and destruction of antibody-coated particles, clearance of immune complexes, lysis of antibody-coated target cells by killer cells (called antibody-dependent cell-mediated cytotoxicity, or ADCC), release of inflammatory mediators, placental transfer and control of immunoglobulin production.

35 Although various Fc receptors and receptor sites have been studied to a certain extent, there is still much which is unknown about their location, structure and functioning.

Summary of the invention

40 According to one aspect of the present invention, there is provided a modified antibody of the class IgG in which at least one selected individual amino acid residue in the constant portion (as herein defined), including at least one amino acid selected from the group comprising amino acid residues 234, 235, 236, 237, 297, 318, 320 and 322, has been replaced by a different residue, altering an effector function of the antibody as compared with unmodified antibody.

45 An effector function of an antibody may be altered by altering, ie enhancing or reducing, the affinity of the antibody for an effector molecule such as an Fc receptor or a complement component. Binding affinity will generally be varied by modifying the effector molecule binding site, and in this case it is appropriate to locate the site of interest and modify at least part of the site in a suitable way. It is also envisaged that an alteration in the binding site on the antibody for the effector molecule need not alter significantly the overall binding affinity but may alter the geometry of the interaction rendering the effector mechanism ineffective as in non-productive binding. It is further envisaged that an effector function may also be altered by modifying a site not directly involved in effector molecule binding, but otherwise involved in performance of the effector function.

55 By altering an effector function of an antibody it may be possible to control various aspects of the immune response, eg enhancing or suppressing various reactions of the immune system, with possible beneficial effects in diagnosis and therapy.

For example, it is known to use monoclonal antibodies for guided localisation of malignant lesions in patients with a number of solid tumours, such as ovarian and testicular cancer. However, their general use

has been limited because several major problems such as false positive false negative as well as non-specific localisation continue to exist. The amounts of radioiodine-labelled tumour-associated monoclonal antibody reaching their target tissues after intra-venous administration in humans are small (Epenetos et al, 1986). One problem is a high non-specific uptake in normal lymph nodes and the rapid catabolism of murine monoclonal antibodies in these studies. The use of human monoclonal antibodies may also give high backgrounds due to non-specific binding to the high affinity receptors (Fc gamma RI) of the lymphatics, liver and spleen. An altered monoclonal antibody which does not bind to this high affinity receptor may improve antibody-guided tumour localisation by enhancing specific tumour uptake of the antibody while decreasing the background due to non-specific binding to FcR.

Ideally, monoclonal antibodies used for therapy of tumours would be radiolabelled or exploit the host's own effector mechanisms. It is not yet clear which of these will be the most significant in vivo for clearance of antibody-coated target cells, but ADCC by mononuclear cells, particularly K cells, seems the most effective (Hale et al, 1985). It may be possible to produce antibodies which react only with certain types of Fc receptor; for example, modified antibodies could be produced which do not bind the high affinity Fc gamma RI of cells of the R.E.S. but, when aggregated on a surface may bind Fc gamma RII expressing cells and trigger ADCC and specifically destroy the target cell.

Production of a modified antibody can be carried out by any suitable technique including techniques that are well known to those skilled in the art. For example an appropriate protein sequence, eg forming part or all of a relevant constant domain, eg C_H2 domain, of an antibody, and include appropriately altered residue(s) can be synthesised and then chemically joined into the appropriate place in an antibody molecule.

Preferably, however, genetic engineering techniques are used for producing an altered antibody. The presently preferred such technique comprises:

- a) preparing a first replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least part of an IgG heavy or light chain, eg the V_H, C_H1 and C_H2 domains of an IgG heavy chain, in which at least one selected individual amino acid residue, including at least one residue from the group comprising amino acid residues 234, 235, 236, 237, 297, 318, 320 and 322, differs from the corresponding residue(s) in unmodified antibody.
- b) if necessary, preparing a second replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes a complementary Ig light or heavy chain;
- c) transforming a cell line with the first or both prepared vectors; and
- d) culturing said transformed cell line to produce an altered antibody.

The present invention also includes vectors used to transform the cell line, vectors used in producing the transforming vectors, cell lines transformed with the transforming vectors, cell lines transformed with preparative vectors, and methods for their production.

Preferably, the cell line which is transformed to produce the antibody of altered effector function is an immortalised mammalian cell line, which is advantageously of lymphoid origin, such as a myeloma, hybridoma, trioma or quadroma cell line. The cell line may also comprise a normal lymphoid cell, such as a B-cell, which has been immortalised by transformation with a virus, such as the Epstein-Barr virus. Most preferably, the immortalised cell line is a myeloma cell line or a derivative thereof.

Although the cell line used to produce the antibody of altered effector function is preferably a mammalian cell line, any other suitable cell line, such as a bacterial cell line or a yeast cell line, may alternatively be used. In particular, it is envisaged that E. coli-derived bacterial strains could be used.

It is known that some immortalised lymphoid cell lines, such as myeloma cell lines, in their normal state secrete isolated Ig light chains. If such a cell line is transformed with the vector prepared in step a) of the process defined above, it will not be necessary to carry out step b) of the process, provided that the normally secreted chain is complementary to the chain encoded by the vector prepared in step a).

However, where the immortalised cell line does not secrete or does not secrete a complementary chain, it will be necessary to carry out step b). This step may be carried out by further manipulating the vector produced in step a) so that this vector encodes not only the heavy chain but also the light chain. Alternatively, step b) is carried out by preparing a second vector which is used to transform the immortalised cell line.

The techniques by which such vectors can be produced and used to transform the immortalised cell lines are well known in the art, and do not form any part of the invention.

In the case where the immortalised cell line secretes a complementary light chain, the transformed cell line may be produced, for example, by transforming a suitable bacterial cell with the vector and then fusing the bacterial cell with the immortalised cell line by spheroplast fusion. Alternatively, the DNA may be directly introduced into the immortalised cell line by electroporation.

The DNA sequence encoding the relevant altered portion of the antibody may be prepared by oligonucleotide synthesis. Alternatively, the DNA encoding the altered portion may be prepared by primer directed oligonucleotide site-directed mutagenesis. This technique in essence involves hybridising an oligonucleotide coding for a desired mutation with a single strand of DNA containing the mutation point and using the single strand as a template for extension of the oligonucleotide to produce a strand containing the mutation. This technique, in various forms, is described by Zoller and Smith, 1982; Zoller and Smith, 1984; Norris et al., 1983; Kramer et al., 1982.

For various reasons, this technique in its simplest form does not always produce a high frequency of mutation. An improved technique for introducing both single and multiple mutations in an M13 based vector has been described by Carter et al., 1985a.

The invention can be applied to antibodies of different species, eg human, rodent (mouse, rat, hamster) etc, and different class. The invention can also be applied to naturally occurring antibodies, chimeric antibodies (eg of the type disclosed in PCT/GB85/00392) or altered antibodies altered in other ways (eg of the type disclosed in GB 2188638).

As one example, work has been carried out on IgG, to alter the binding affinity for the receptor known as Fc gamma R1.

In man, and in mouse, three Fc gamma receptors have been partially characterised: Fc gamma R1, Fc gamma RII, and Fc gamma R₁₀, and these are expressed on distinct but overlapping haematopoietic cell types (Anderson and Looney, 1986). Furthermore, these different receptors have differing affinities for IgG subclasses. As mentioned above, binding of antibody to these receptors on cell surfaces triggers a number of important and diverse biological responses. It is not known which receptor, if any, is primarily responsible for which effect, but evidence suggests that it is the low affinity receptors which are relevant for these physiological effects. The receptors in man and mouse have been proposed as homologues or, a number of physical criteria. Cloning and sequencing of the low affinity Fc gamma RII from both sources has confirmed this prediction (Lewis et al 1986, Ravetch et al 1986). The high affinity receptor Fc gamma R1 has been studied extensively and in both man and mouse binds monomeric IgG (man = IgG1 and IgG3; mouse = IgG2a) and is found on the same cell types.

The Fc region of IgG comprises two constant domains, C_H2 and C_H3, as shown in Figure 1. As with the mouse system much effort has gone into the determination of the contribution of each of the two domains, C gamma 2 and C gamma 3, to the interaction. Isolated C_H3 domains, (pFc' fragments) were reported to have no inhibitory activity on the formation of monocyte rosettes (Abramson et al 1970). But other reports have shown that this fragment was capable of inhibiting Fc gamma R1 binding (Barnett-Foster et al 1980) indicating that the C gamma 3 domain was involved in binding human Fc gamma R1. This view became predominant until Woof and colleagues demonstrated that this inhibitory activity could be removed by extensive purification of the pFc' fraction by passage over protein A and anti-L chain columns. These purified samples showed no inhibition of monomer binding (Woof et al 1984). Additionally, the ability of monoclonal antibodies directed against epitopes on the C_H3 domain to interact with FcR bound antibody, but not those to epitopes on C_H2, is consistent with a binding site on the C_H2 domain (Partridge et al, 1986).

In a comprehensive study of the high affinity receptor for human IgG on human monocytes (Fc gamma R1) Woof, Burton and colleagues also localised the binding-site to the C_H2 domain of human IgG1 (Woof et al, 1984; Partridge et al, 1986). A range of IgG subclasses from different species, as well as fragments of human immunoglobulin, were tested for their ability to inhibit the interaction between human IgG and human monocytes in a direct-binding microassay.

IgGs were grouped into those found to exhibit tight, intermediate or weak binding to the FcR on human monocytes (Fc gamma R1). By comparison of the amino-acid sequences in these different affinity groups a potential monocyte binding site in the hinge-link region (Leu234 -Ser239) was proposed, with possible involvement of the two beta-strands and joining bend formed by the residues Gly316 - Lys338 (Woof et al, 1986). The latter region had already been proposed as the C1q binding site (Burton et al, 1980). The human Fc gamma R1 receptor binds human IgG1 and mouse IgG2a as a monomer, but the binding of mouse IgG2b is 100-fold weaker (Woof et al, 1986). A comparison of the sequence of these proteins in the hinge-link region shows that the sequence (234 to 238) Leu-Leu-Gly-Gly-Pro in the strong binders becomes Leu-Glu-Gly-Gly-Pro in mouse gamma 2b.

In an attempt to alter the binding affinity the substitution Glu235 by Leu was made in the mouse IgG2b heavy chain. The numbering of the residues in the heavy chain is that of the EU index (see Kabat et al., 1983). The normal mouse antibody does not bind to human Fc gamma R1, but by changing residue 235 from glutamic acid to leucine, eg by site directed mutagenesis, affinity for the human Fc gamma R1 is increased by over 100-fold. The magnitude of the increase in affinity was much greater than could have been expected and suggests that single amino acid changes in this region could be used to produce altered

antibodies more suited to a range of in vivo applications in man and other animals. This change does not alter other Ig binding sites such as for complement component C1q.

It should also be possible to alter the affinity of Fc gamma R1 binding by replacing the specified residue with a residue having an inappropriate functionality on its sidechain, or by introducing a charged functional group, such as Glu or Asp, or perhaps an aromatic non-polar residue such as Phe, Tyr or Trp.

These changes would be expected to apply equally to the murine, human and rat systems given the sequence homology between the different immunoglobulins. It has been shown that in human IgG3, which binds to the human Fc gamma R1 receptor, changing Leu 235 to Glu destroys the interaction of the mutant for the receptor. The binding site for this receptor can thus be switched on or switched off.

Mutations on adjacent or close sites in the hinge link region (eg replacing residues 234, 236 or 237 by Ala) indicate that alterations in residues 234, 235, 236 and 237 at least affect affinity for the Fc gamma R1 receptor.

Hence in a preferred aspect the present invention provides a modified antibody of the class IgG having an altered Fc region with altered binding affinity for Fc gamma R1 as compared with the unmodified antibody, having a modification at least at residue 234, 235, 236 or 237.

Affinity for other Fc receptors can be altered by a similar approach, for controlling the immune response in different ways.

As a further example, work has also been carried out to alter the lytic properties of Ig following binding of the C1 component of complement.

The first component of the complement system, C1, actually comprises three proteins known as C1q, C1r and C1s which bind tightly together. It has been shown that C1q is responsible for binding the three protein complex to Ig.

It has been shown that isolated Fc fragment inhibits the interaction of C1q with an Ig (Yasmeeri et al., 1976).

It has also been shown that the binding of C1q is dependent on ionic strength, suggesting that ionic interactions are involved.

It is possible to cleave the C_H3 domain from the remainder of an Ig molecule, and it has been shown that deletion of the C_H3 domain does not abolish C1q binding activity (Colomb and Porter, 1975).

It is also possible to isolate the C_H2 domain from Igs. It has been shown that such isolated C_H2 domains have the same binding affinity for C1q as do isolated Fc fragments (Isenman et al., 1975).

From this, it has been inferred that the binding site for C1q is located in the C_H2 domain of the Ig. Various attempts have been made to identify the particular amino acid residues in the C_H2 domain involved in C1q binding. In a first approach, synthetic peptides corresponding to short sections of the C_H2 domain were tested for inhibition of C1q binding. This identified two possible binding sites (Boakle et al., 1975 and Lukas et al., 1981).

In a second approach, a comparison of the sequences of several Ig C_H2 domains was made in conjunction with studies of their three dimensional structure. This led to the identification of two further proposals for the site of C1q binding (Brunhouse and Cebra, 1979 and Burton et al., 1980).

It has now been found that the C1q binding activity of an antibody can be altered by providing the antibody with an altered C_H2 domain in which at least one of the amino acid residues 318, 320 and 322 of the heavy chain has been changed to a residue having a different side chain.

The numbering of the residues in the heavy chain is that of the EU index (see Kabat et al., 1983).

The present inventors have discovered that, in a specific C1q-binding Ig referred to below, by changing any one of residues 318 (Glu), 320 (Lys) and 322 (Lys), to Ala, it is possible to abolish C1q binding.

Moreover, by making mutations at these residues, it has been shown that C1q binding is retained as long as residue 318 has a hydrogen-bonding side chain and residues 320 and 322 both have a positively charged side chain.

The Applicants believe that these three residues are probably involved directly in the binding C1q to IgG. However, it is also possible that these residues are not directly involved in physical contact with C1q. These residues may help one C_H2 domain to pack against an adjacent domain in an IgG aggregate, thus producing the at least two molecules of IgG together which are required for C1q binding. If this is the case, the C1q may be in direct contact with the IgG in an entirely different area. The Applicants, however, do not wish to be in any way limited to either of these theories.

Altering residue 333 (Glu), which is close to the three specified residues, or residue 253 (Ile), which is distant from the three specified residues, does not alter C1q binding activity, even though previous studies have implicated residue 333 (Glu) in C1q binding.

It is to be noted that residues 318, 320 and 322 are highly conserved in mouse and human IgGs which are complement binding.

It has also been shown that alteration of the three specified residues only alters the C1q binding activity, and does not alter antigen binding activity protein A binding activity (protein A binds to the C_H2/C_H3 interface), or the ability of the Fc to bind to mouse macrophages.

It is believed that the process of the present invention can be used to abolish C1q binding activity by replacing any one of the three specified residues with a residue having an inappropriate functionality on its side chain. It is not necessary to replace the ionic residues only with Ala to abolish C1q binding. It will also be possible to use other alkyl-substituted non-ionic residues, such as Gly, Ile, Leu, or Val, or such aromatic non-polar residues as Phe, Tyr, Trp and Pro in place of any one of the three residues in order to abolish C1q binding. It will also be possible to use such polar non-ionic residues as Ser, Thr, Cys, and Met in place of residues 320 and 322, but not 318, in order to abolish C1q binding activity.

It may be that the side chains on ionic or non-ionic polar residues will be able to form hydrogen bonds in a similar manner to the bonds formed by the Glu residue. Therefore, replacement of the 318 (Glu) residue by a polar residue may modify but not abolish C1q binding activity.

It has further been shown that replacing residue 297 (Asn) with Ala results in removal of lytic activity while only slightly reducing (about three fold weaker) affinity for C1q. It is thought this is because the alteration destroys the glycosylation site and that the presence of carbohydrate is required for complement activation. Any other substitution at this site will also destroy the glycosylation site.

Further, the mutation Lys 320 to Gln has an affinity for C1q only slightly weaker than the wild type but is non lytic. This indicates that good C1q binding may be insufficient for lysis and that perhaps a precise orientation of C1q is required.

All antibody isotypes sequenced to date possess the C1q binding motif, or a closely related motif which is effective in binding C1q when it is transplanted into the mouse IgG2b antibody. Clearly there must be further determinants for lysis. For example, antibody isotypes with short hinges and low segmental flexibility are non-lytic (Oi et al. 1984) suggesting that (a) the interaction of C1q with the motif may be sterically blocked due to close approach of the Fc by the Fab arms (Leatherbarrow et al., 1985) or (b) the interaction of C1q and antibody requires an exact alignment for lysis and therefore requires some flexibility per se.

The present invention is now described, by way of example only, with reference to the accompanying drawings, in which:-

Figure 1 illustrates the structure of an Ig;

Figure 2 illustrates the sequence of cloning steps used to produce an antibody of altered Fc gamma R1 binding activity;

Figure 3 shows the sequence of mouse IgG gamma 2b gene;

Figure 4 is a graph illustrating inhibition of ¹²⁵I-labelled pooled human IgG binding to high affinity receptors on U937 cells by mouse gamma 2b immunoglobulins;

Figure 5 is a Scatchard plot of ¹²⁵I-EL235 binding to U937 high affinity receptors;

Figure 6 shows the nucleotide sequence and protein sequence of the human gamma 3 gene; and

Figure 7 shows the nucleotide sequence encoding the C_H2 domain of mouse IgG2b antibody with mutants and the sequences of the oligonucleotides used to construct some of the mutants referred to below.

The following concerns experiments on mouse IgG2b to alter the affinity thereof for human Fc gamma R1.

DNA encoding the variable and constant region exons of antibodies can be manipulated in vitro and reintroduced into lymphoid cell lines (Neuberger, 1985). Using vectors based on pSV-gpt (Mulligan & Berg 1981) and the Ig heavy chain promoter/enhancer, antibodies can be expressed and secreted. One such vector, pSV-VNP 2b (Neuberger and Williams 1985) encodes a variable domain which binds nitrophenylacetyl (NP) and the constant domains of the natural mouse IgG2b antibody. The antibody produced using this vector does not bind to human Fc gamma R1.

Part of the structure of the pSV-VNP 2b vector is shown in Figure 2(a). The vector was partially digested with SacI and a fragment containing both the C_H2 and C_H3 domains was cloned into plasmid M13K19 (Carter et al, 1985a) as shown in Figure 2(b).

The SacI site at the N-terminal end of the C_H3 domain was removed by site directed mutagenesis with an oligonucleotide which retains the amino acid sequence at this N-terminal end.

A point mutation in the C_H2 domain was then produced using a synthetic oligonucleotide as shown in Figure 3, in the region indicated between bases 956 and 975 and marked EL235. Further details of the construction of the mutation is given below. The mechanism of the point mutation is shown in Figure 2(c).

The mutant C_H2-C_H3 fragments were recloned into the pSV-VNP 2b vector to replace the wild type C_H2-C_H3 domains. The mutant pSV-VNP 2b vectors were incorporated into J558L, cultured to produce antibody and the antibody mutant known as EL235 was purified on NIP-Sepharose.

Construction of mutations in the C gamma 2 exon

Mutations were constructed in the M13B19-C gamma 2/C gamma 3 as in Carter et al (1985a). The principles and methods are described in detail in Carter et al, (1985b), and Duncan.

The mutant EL235 was assayed by inhibition of binding of human IgG as well as by direct binding to a human monocyte cell line (Woof et al, 1984; 1986). Inhibition of binding of monomeric ^{125}I -labelled normal pooled human IgG to high affinity Fc receptors on a human monocyte cell line, U937, was measured in a quantitative microassay system in which free and cell-bound label were separated by centrifugation through a water-immiscible oil. The binding of wild-type gamma 2b and the mutant EL235 were compared by competition of labelled polyclonal human IgG. Figure 4 shows the inhibition curves for this experiment. In Figure 4 empty circles represent wild type and solid circles mutant EL235. The result has been normalised such that the fractional binding of ^{125}I -IgG = 1 in the absence of inhibitors. The mutant inhibits the binding of human IgG1; the wild type protein showed no inhibitory activity. Direct binding of radiolabelled mutant EL235 to U937 cells gives a binding constant of $3.13 \times 10^8 \text{ M}^{-1}$ (Figure 5), very similar to the value for pooled human IgG in the same experiment.

Figure 5 is a typical Scatchard plot of ^{125}I -EL235 binding to U937 high affinity FC receptors. The number of moles of ^{125}I -EL235 bound per mole of cells, r , was calculated using the following relationship:-

$$r = \frac{6 \times 10^{23} \times \text{IgG2b}}{\text{no. cells/L}}$$

where IgG2b is the concentration of bound ^{125}I -EL235. A represents the concentration of free ^{125}I -EL235. The coefficient of correlation of the plot was 0.95.

Thus a point mutation altered the binding affinity of mouse IgG2b for human Fc gamma R1 by greater than 100-fold.

Mutations were made in the human gamma 3 gene (Huck et al., 1985); the Hind III - SpHI fragment was first subcloned into M13 mp19 after attaching BamHI linkers. Then synthetic oligonucleotides were used as described previously to make the mutations:

234 Leu to Ala

235 Leu to Glu

236 Gly to Ala

237 Gly to Ala

as indicated on Figure 6.

The BamHI fragment was attached to a Hind III - BamHI fragment encoding the variable domain of the B18 antibody (as in Neuberger et al., 1984 and 1985) and cloned for expression into a pSVgpt vector.

The properties of the recombinant antibodies in binding in Fc gamma R1 were determined indirectly in a competition assay as described in connection with Figure 4. Table 1 shows the concentration of antibody required to inhibit the binding of ^{125}I labelled pooled human IgG to U937 cells.

Table 1

	<u>I₅₀</u> (M)
Wild type (Leu 234, Leu 235, Gly 236, Gly 237)	10 ⁻⁸
Mutants	
Ala 234	4 x 10 ⁻⁸
Glu 235	greater than 10 ⁻⁶
Ala 236	3 x 10 ⁻⁸
Ala 237	3 x 10 ⁻⁷

The table gives the approximate values of I₅₀ (ie the concentration of IgG3 at which the fractional binding of ¹²⁵I labelled pooled human IgG is 0.5).

These findings have important implications for the use of antibodies, both murine and human, in diagnostics and therapy, as discussed above.

The present results show that Fc gamma R1 receptor can be selectively switched on or off, and this might be of great use in the preparation of antibodies for in vivo diagnosis or therapy of humans as well as other animals.

Similar experiments were carried out at mouse IgG2b to alter lytic activity following binding of C1q. Further mutants of the pSV-VNP 2b vector were produced using the procedure described above, with point mutations being produced in the C_H2 domain using synthetic oligonucleotides as shown in Figure 7, and antibodies produced as previously described.

Antibody produced using the pSV-VNP 2b vector, with wild type C_H2-C_H3 domains binds C1q (see Table 2).

The ability of the resulting purified antibodies to lyse specifically NIP-kephalin derivatised sheep red blood cells (Weltzien et al., 1984) was tested in a quantitative haemolysis microassay (Young et al., 1986). The results of the test are shown in Table 2. The titre in ug/ml antibody represents the amount of antibody required for 50% lysis after 30 minutes at 37° C.

A number of the mutant antibodies were tested for affinity for radiolabelled C1q (Leatherbarrow and Dwek, 1984) after aggregating the anti-NP antibodies on NP-Affigel. The results are shown also in Table 2.

Table 2

5	IgG	titre (ug/ml)	Affinity nM
	MolG2b	3	10
10	MolGM	0.15	-
	MolG1	X	-

Irrelevant Mutations of MolG2b

20	Pro 331 - Ala	3	-
	Pro 331 - Gly	-	12
25	Glu 333 - Ala	3	12
	Thr 335 - Ala	3	10
	Ser 337 - Ala	3	11
	Glu 283 - Ala	3	-
30	His 285 - Ala	3	12
	His 290 - Ala	3	11
	Glu 294 - Ala	3	-
35	Glu 235 - Ala	3	-
	Lys 248 - Ala	3	-
	Ile 253 - Ala	3	9
40	Ser 267 - Ala	3	-
	Asp 270 - Ala	3	-
	Gln 274 - Ala	3	-
	Lys 317 - Ala	3	-
45	Lys 236 - Ala	3	-
	Lys 340 - Ala	3	-

50

55

Mutations of MolgG2b which abolish lytic activity

5	Glu 318 - Val	x	-
	Glu 318 - Ala	x	greater than 300
	Lys 320 - Ala	x	greater than 300
10	Lys 320 - Gln	x	13
	Lys 322 - Ala	x	greater than 300
	Lys 322 - Gln	x	-
15	Asn 297 - Ala	x	31

Mutations of MolgG2b which conserve lytic activity

20			
	Glu 318 - Thr	3	12
25	Lys 320 - Arg	3	11
	Lys 322 - Arg	3	11

30 Antibodies with the V_{NP} domain attached to human IgG1 and mouse IgG1 were kindly supplied by Dr M Bruggemann and Mr P T Jones respectively.

Mutants Glu318-Ala, Lys320-Ala and Lys322-Ala have a dramatically reduced affinity (Table 2). However, they retain binding for the NP hapten and protein A (which binds at the C_H2-C_H3 interface). This suggests that the loss of C1q binding is not due to major structural change in the antibody. Mutations in adjacent residues (Glu333-Ala) or distant residues (Ile253-Ala) retain C1q affinity.

35 The results suggest that a surface patch defined by the side chains of residues 318, 320 and 322 determine whether an IgG will interact with C1q. These residues are highly conserved in human and mouse IgGs, indicating that alterations of side chains at these three locations can be used to construct therapeutically useful variants of human C_H2 domains which do not activate complement, or which have an enhanced affinity for complement.

Evidence that this surface patch is the complete binding site for C1q comes from a polypeptide mimic containing the Glu X Lys X Lys motif which proved to inhibit C1q lysis in a model system. This work is described in a copending PCT application No. of Research Corporation entitled "Complement Binding Peptide" filed on the same date as this application.

45 It will be appreciated that the present invention has been described above purely by way of illustration and that variations and modifications can be made without departing from the scope of the invention.

REFERENCES

- 50 Abramson, N., Gelfand, E.W., Jandl, J.H., and Rosen, F.S. (1970) J. Exp. Med. 132, 1207.
 Anderson, C.L., and Looney R.J. (1986) Immunol. Today, 7, 264.
 Barnett-Foster, D.E., Dorrington, K.J., and Painter, R.H. (1980) J. Immunol. 124, 2186
 Boakle et al., (1975) Nature, 282, 742-743
 Brunhouse and Cebra, (1979) Molec. Immun., 16, 907-917.
 55 Burton et al., (1980) Nature, 288, 338-344.
 Burton, D.R. (1985) Molec. Immunol. 22, 161.
 Burton, D.R., Boyd, J., Brampton, A., Easterbrook-Smith, S., Emanuel, E.J., Novotny, J., Rademacher, T.W., van Schravendijk, J.R., Sternberg, M.J.E., and Dwet, R.A. (1980) Nature 288, 338.

- Carter, P., Bedouelle, H., and Winter, G. (1985a) *Nucleic Acids Res.* 13, 4431-4443.
- Carter, P., Bedouelle, H., Waye, M.Y., and Winter, G. (1985b) In: *Oligonucleotide-site-directed mutagenesis in M13*. Anglian Biotechnology Limited, Colchester, England.
- Colomb and Porter (1975) *Biochem J.*, 145, 177-183.
- 5 Duncan A.R., University of Cambridge PhD Thesis (to be published).
- Epenetos, A., Snook, D., Durbin, H., Johnson, P., and Tayler-Papadimitriou, J. (1986) *Cancer Res.* 46,3183.
- Hale, G., Clark, M., and Waldmann, H. (1985) *J. Immunol.* 1134, 3056.
- Hale, G., and Waldmann, H. (1985) in *Hybridoma Technology in the Biosciences and Medicine*. T. Springer, ed. Plenum Press, New York.
- 10 Huber, H., and Fudenberg, H.H. (1968) *Int. Arch. Allergy Appl. Immunol.* 34, 18.
- Huck et al., (1985) *Nuc. Acids Res* 14, 1779-1788.
- Isenman et al., (1975) *J. Immun.*, 114, 1726-1729.
- Kabat et al., (1983) "Sequences of Proteins of Immunological Interest", US Dept. Health and Human
- 15 Services.
- Kalofonos, H.P., and Epenetos, A. (1986) *Cancer Treatment Reviews* 13,243.
- Kramer et al., (1982) *Nuc. Acides Res.*, 10, 6475-6485.
- Leatherbarrow and Dweck (1984) *Molec Immun.*, 21, 321-327.
- Leatherbarrow, R.J., Rademacher, T.W., Dwek, R.A., Woof, J.M., Clark, A., Burton, D.R., Richardson, N.,
- 20 and Feinstein, A. (1985) *Molec. Immun.* 22,407-415.
- Lewis, V.A., Koch, T., Plutner, H., and Mellman, I. (1986) *Nature* 324, 372.
- Lukas et al., (1981) *J. Immun.*, 127, 2555-2560.
- Mulligan, R.C., and Berg, P. (1981) *Proc. Natl. Acad. Sci. USA* 78,2072.
- Neuberger, M.S., and Williams, G.T. (1986) *Phil. Trans. R. Soc. Lond. A* 317, 425-432.
- 25 Neuberger, M.S., Williams, G.T., Mitchell, E.B., Jouhal, S.S., Flanagan, J.G., and Rabbitts, T.H. (1985) *Nature* 314, 268.
- Neuberger, M.S., Williams, G. T. and Fox, R.O. (1984) *Nature*, 312, 604-608
- Norris et al., (1983) *Nuc. Acids Res.*, 11, 5103-5112.
- Oi, V.T., Minh-Vuong, T., Hardy, R.R., Reidler, J., Dangl, J.L., Herzenberg L.A. and Stryer (1984) *Nature*,
- 30 307, 136-140.
- Partridge, L.J., Woof, J.M., Jefferis, R., and Burton, D.R. (1986) *Molec. Immunol.* 23, 1365.
- Ravetch, J.V., Luster, A.D., Weinshank, R., Kochan, A., Pavlovic, D.A., Portnoy, J., Hulmes, J., Pan, Y.M.C., Unkeless, J. (1986) *Science* 234, 718.
- Suggs, S.V., Hirose, T., Miyake, T., Kawashima, E.H., Johnson, M.J., Itakura, K., and Wallace, R.B.
- 35 (1981) In: *Developmental Biology using Purified Genes* (D. Brown, ed.) Academic Press, New York.
- Weltzien et al., (1984) *Molec. Immunol.*, 21 801.
- Woof, J.M., Jafaar, M.I., Jefferis, R., and Burton, D.R. (1984) *Molec. Immun.* 21, 523.
- Woof, J.M., Partridge, L.J., Jefferis, R., and Burton D.R. (1986) *Molec. Immun.* 23, 319.
- Yasmeen et al., (1976) *J. Immun.*, 116, 518-522.
- 40 Young et al., (1986) *Anal. Biochem.*, 154, 649-654.
- Zoller and Smith (1982) *Nuc. Acids Res.*, 10, 6487-650.
- Zoller and Smith (1984) *DNA*, 3, 497-488.

Claims

- 45
1. A modified antibody of the class IgG in which at least one selected individual amino acid residue in the constant portion (as herein defined), including at least one amino acid selected from the group comprising amino acid residues 234, 235, 236, 237, 297, 318, 320 and 322, has been replaced by a different residue altering an effector function of the antibody as compared with unmodified antibody.
 - 50
 2. An antibody according to claim 1, wherein the antibody has altered affinity for an effector molecule as compared with unmodified antibody.
 - 55
 3. An antibody according to claim 1 or 2, having an altered Fc region with altered binding affinity for an Fc receptor as compared with unmodified antibody, in which at least one amino acid selected from the group comprising amino acid residues 234, 235, 236, and 237, has been replaced by a different residue.

4. An antibody according to claim 3, having an altered binding affinity for Fc gamma R1 receptor as compared with unmodified antibody.
5. An antibody according to claim 3 or 4, wherein residue 235 has been replaced by Glu.
6. An antibody according to claim 3 or 4, wherein at least one of residues 234, 236 and 237 has been replaced by Ala.
7. An antibody according to claim 1 or 2, having an altered Fc region with altered binding affinity for C1q as compared with unmodified antibody, in which at least one amino acid selected from the group comprising amino acid residues 318, 320 and 322, has been replaced by a different residue.
8. An antibody according to claim 7, wherein at least one of the residues 318, 320 and 322 has been changed to Ala, reducing C1q binding affinity.
9. An antibody according to claim 7, wherein residue 318 has been changed to Val.
10. An antibody according to claim 7, wherein residue 322 has been changed to Gln.
11. An antibody according to claim 1, having an altered Fc region with altered lytic properties as compared with unmodified antibody, in which at least amino acid residue 297 has been replaced by a different residue.
12. An antibody according to claim 11, where residue 297 has been replaced by Ala.
13. An antibody according to claim 1, comprising rodent or human IgG.
14. An antibody according to claim 1, wherein the antibody is a natural antibody, a chimaeric antibody or an altered antibody.
15. A process for altering an effector function of an antibody of the class IgG for an effector molecule, comprising replacing at least one selected individual amino acid residue, in the constant portion (as herein defined), including at least one amino acid from the group comprising amino acid residues 234, 235, 236, 237, 297, 318, 320 and 322, with a different residue, altering an effector function of the antibody as compared with unmodified antibody.
16. A method of producing a modified antibody of the class IgG with an altered effector function as compared with unmodified antibody, comprising:-
 - a) preparing a first replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least part of the constant portion of an Ig heavy chain and in which at least one selected individual amino acid residue including at least one residue from the group comprising amino acid residues 234, 235, 236, 237, 297, 318, 320 and 322, differs from the corresponding residue(s) in unmodified antibody;
 - b) if necessary, preparing a second replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes a complementary Ig light chain;
 - c) transforming a cell line with the first or both prepared vectors; and
 - d) culturing said transformed cell line to produce a modified antibody.

Patentansprüche

1. Ein modifizierter Antikörper der Klasse IgG, bei dem mindestens ein ausgewählter einzelner Aminosäurerest im konstanten Teil (wie hier definiert), einschließlich mindestens einer Aminosäure, die aus einer Gruppe ausgewählt ist, die die Aminosäurereste 234, 235, 236, 237, 297, 318, 320 und 322 enthält, durch einen anderen Rest ersetzt worden ist, der eine Effektorfunktion des Antikörpers im Vergleich mit einem nichtmodifizierten Antikörper verändert.
2. Ein Antikörper nach Anspruch 1, bei dem der Antikörper die Affinität zu einem Effektormolekül im Vergleich zu einem nichtmodifizierten Antikörper verändert hat.

3. Ein Antikörper nach Anspruch 1 oder 2, der einen veränderten Fc-Bereich mit veränderter Bindungsaffinität zu einem Fc-Rezeptor im Vergleich zum nichtmodifizierten Antikörper aufweist, in dem mindestens eine Aminosäure, die aus der Gruppe ausgewählt ist, die die Aminosäurereste 234, 235, 236 und 237 enthält, durch einen Rest ersetzt worden ist.
4. Ein Antikörper nach Anspruch 3, der eine veränderte Bindungsaffinität zum Fc-gamma-R1-Rezeptor im Vergleich zu einem nichtmodifizierten Antikörper aufweist.
5. Ein Antikörper nach Anspruch 3 oder 4, bei dem der Rest 235 durch Glu ersetzt worden ist.
6. Ein Antikörper nach Anspruch 3 oder 4, bei dem mindestens einer der Reste 234, 236 und 237 durch Ala ersetzt worden ist.
7. Ein Antikörper nach Anspruch 1 oder 2, der einen veränderten Fc-Bereich mit veränderter Bindungsaffinität zu C1q im Vergleich mit einem nichtmodifizierten Antikörper aufweist, in dem mindestens eine Aminosäure, die aus der Gruppe ausgewählt ist, die die Aminosäurereste 318, 320 und 322 enthält, durch einen anderen Rest ersetzt worden ist.
8. Ein Antikörper nach Anspruch 7, bei dem mindestens einer der Reste 318, 320 und 322 unter Reduzierung der C1q-Bindungsaffinität in Ala geändert worden ist.
9. Ein Antikörper nach Anspruch 7, bei dem der Rest 318 in Val geändert worden ist.
10. Ein Antikörper nach Anspruch 7, bei dem der Rest 322 in Gln geändert worden ist.
11. Ein Antikörper nach Anspruch 1, der einen veränderten Fc-Bereich mit veränderten lytischen Eigenschaften im Vergleich zu einem nichtmodifizierten Antikörper aufweist, in dem mindestens der Aminosäurerest 297 durch einen anderen Rest ersetzt worden ist.
12. Ein Antikörper nach Anspruch 11, bei dem der Rest 297 durch Ala ersetzt worden ist.
13. Ein Antikörper nach Anspruch 1, der Nagetier- oder Human-IgG enthält.
14. Ein Antikörper nach Anspruch 1, wobei der Antikörper ein natürlicher, schimärischer oder ein veränderter Antikörper ist.
15. Ein Verfahren zum Verändern einer Effektorfunktion eines Antikörpers der Klasse IgG für ein Effektormolekül, bei dem mindestens ein ausgewählter einzelner Aminosäurerest im konstanten Teil (wie hier definiert), einschließlich mindestens einer Aminosäure, die aus einer Gruppe ausgewählt ist, die die Aminosäurereste 234, 235, 236, 237, 297, 318, 320 und 322 enthält, durch einen anderen Rest ersetzt wird, der eine Effektorfunktion des Antikörpers im Vergleich mit einem nichtmodifizierten Antikörper verändert.
16. Ein Verfahren zum Zubereiten eines modifizierten Antikörpers der Klasse IgG mit einer im Vergleich mit einem nichtmodifizierten Antikörper veränderten Effektorfunktion mit
 - a) Zubereiten eines ersten, replizierbaren Expressionsvektors, einschließlich eines geeigneten Promotors, der arbeitsfähig mit einer DNA-Sequenz verknüpft ist, die mindestens einen Teil des konstanten Teils einer Ig-H-Kette codiert, und in dem mindestens ein ausgewählter, einzelner Aminosäurerest, einschließlich mindestens eines Restes aus der Gruppe, die die Aminosäurereste 234, 235, 236, 237, 297, 318, 320, 322 enthält, sich von dem/den korrespondierenden Rest(en) in einem nichtmodifizierten Antikörper unterscheidet;
 - b) wenn notwendig, Zubereiten eines zweiten, replizierbaren Expressionsvektors, einschließlich eines geeigneten Promotors, der arbeitsfähig mit einer DNA-Sequenz verknüpft ist, die eine komplementäre Ig-L-Kette codiert;
 - c) Transformation einer Zelllinie mit dem ersten oder mit beiden zubereiteten Vektoren;
 - d) Kultivieren der transformierten Zelllinie zum Zubereiten eines modifizierten Antikörpers.

Revendications

1. Anticorps modifié de la classe IgG dans lequel au moins un résidu d'acide aminé individuel sélectionné dans la portion constante (telle que définie ici), y compris au moins un acide aminé sélectionné parmi le groupe comprenant les résidus d'acides aminés 234, 235, 236, 237, 297, 318, 320 et 322, a été remplacé par un résidu différent modifiant une fonction effectrice de l'anticorps comparé à l'anticorps non modifié.
2. Anticorps selon la revendication 1, caractérisé en ce que l'anticorps a une affinité pour une molécule effectrice modifiée comparé à l'anticorps non modifié.
3. Anticorps selon la revendication 1 ou 2, ayant une région Fc modifiée avec une affinité de liaison pour un récepteur Fc modifiée comparé à l'anticorps non modifié, dans lequel au moins un acide aminé sélectionné parmi le groupe comprenant les résidus d'acides aminés 234, 235, 236, et 237, a été remplacé par un résidu différent.
4. Anticorps selon la revendication 3, ayant une affinité de liaison pour le récepteur Fc gamma R1 modifiée comparé à l'anticorps non modifié.
5. Anticorps selon la revendication 3 ou 4, caractérisé en ce que le résidu 235 a été remplacé par Glu.
6. Anticorps selon la revendication 3 ou 4, caractérisé en ce qu'au moins l'un des résidus 234, 236 et 237 a été remplacé par Ala.
7. Anticorps selon la revendication 1 ou 2, ayant une région Fc modifiée avec une affinité de liaison pour C1q modifiée comparé à l'anticorps non modifié, dans lequel au moins un acide aminé sélectionné parmi le groupe comprenant les résidus d'acides aminés 318, 320 et 322, a été remplacé par un résidu différent.
8. Anticorps selon la revendication 7, caractérisé en ce qu'au moins l'un des résidus 318, 320 et 322 a été changé en Ala, réduisant l'affinité de liaison de C1q.
9. Anticorps selon la revendication 7, caractérisé en ce que le résidu 318 a été changé en Val.
10. Anticorps selon la revendication 7, caractérisé en ce que le résidu 322 a été changé en Gln.
11. Anticorps selon la revendication 1, ayant une région Fc modifiée avec des propriétés lytiques modifiées comparé à l'anticorps non modifié, dans lequel au moins le résidu d'acide aminé 297 a été remplacé par un résidu différent.
12. Anticorps selon la revendication 11, caractérisé en ce que le résidu 297 a été remplacé par Ala.
13. Anticorps selon la revendication 1, comprenant l'IgG de rongeur ou humaine.
14. Anticorps selon la revendication 1, caractérisé en ce que l'anticorps est un anticorps naturel, un anticorps chimérique ou un anticorps modifié.
15. Procédé de modification d'une fonction effectrice d'un anticorps de la classe IgG pour une molécule effectrice, comprenant le remplacement d'au moins un résidu d'acide aminé individuel sélectionné, dans la portion constante (telle que définie ici), y compris au moins un acide aminé issu du groupe comprenant les résidus d'acides aminés 234, 235, 236, 237, 297, 318, 320 et 322, par un résidu différent, modifiant une fonction effectrice de l'anticorps comparé à l'anticorps non modifié.
16. Méthode de production d'un anticorps modifié de la classe IgG avec une fonction effectrice modifiée comparé à l'anticorps non modifié, comprenant:-
 - a) la préparation d'un premier vecteur d'expression réplcatif renfermant un promoteur approprié lié de manière fonctionnelle A une séquence d'ADN codant pour au moins une partie de la portion constante d'une chaîne lourde d'Ig et dans lequel au moins un résidu d'acide aminé individuel

EP 0 307 434 B1

sélectionné, y compris au moins un résidu issu du groupe comprenant les résidus d'acides aminés 234, 235, 236, 237, 297, 318, 320 et 322, diffère du(des) résidu(s) correspondant(s) dans l'anticorps non modifié;

b) le cas échéant, la préparation d'un deuxième vecteur d'expression réplcatif renfermant un promoteur approprié lié de manière fonctionnelle à une séquence d'ADN codant pour une chaîne légère complémentaire d'Ig;

c) la transformation d'une lignée cellulaire à l'aide du premier ou des deux vecteurs préparés; et

d) la culture de ladite lignée cellulaire transformée pour produire un anticorps modifié.

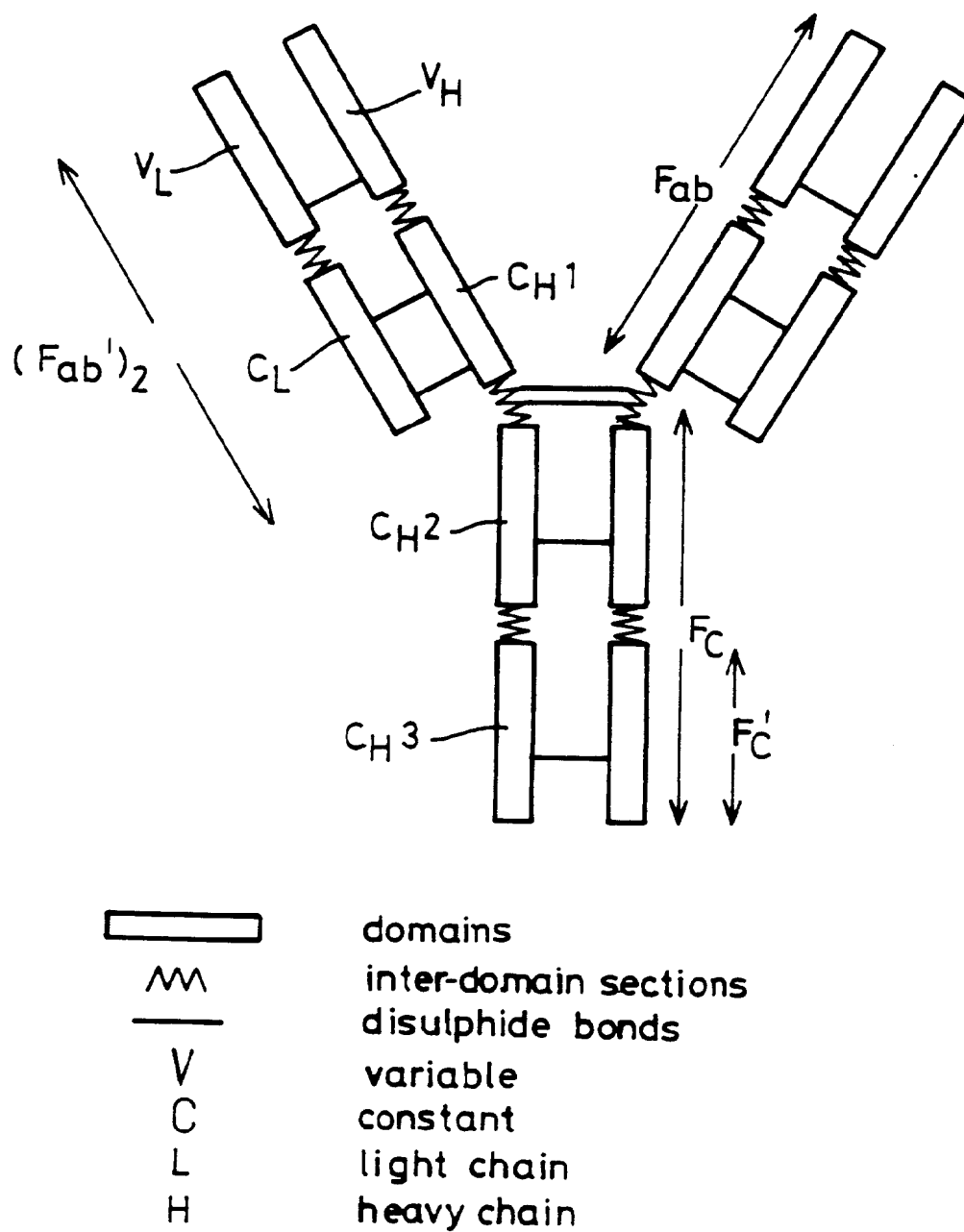


Fig.1

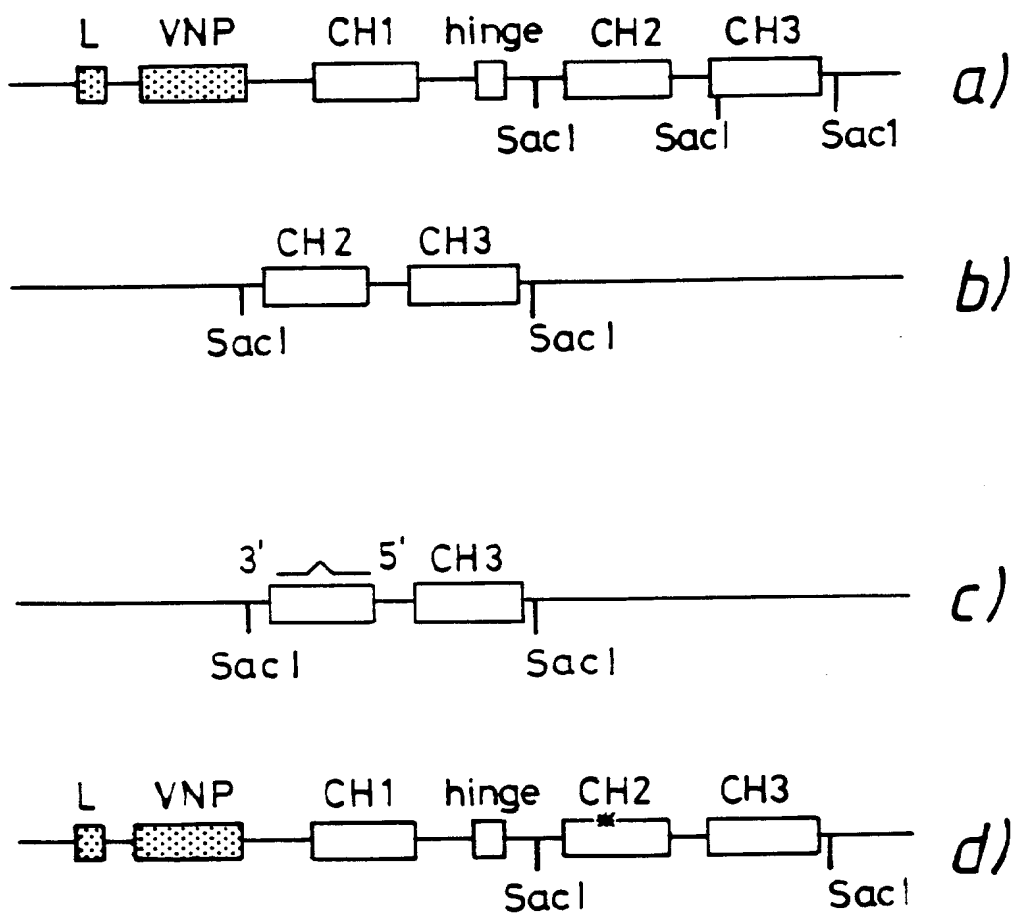
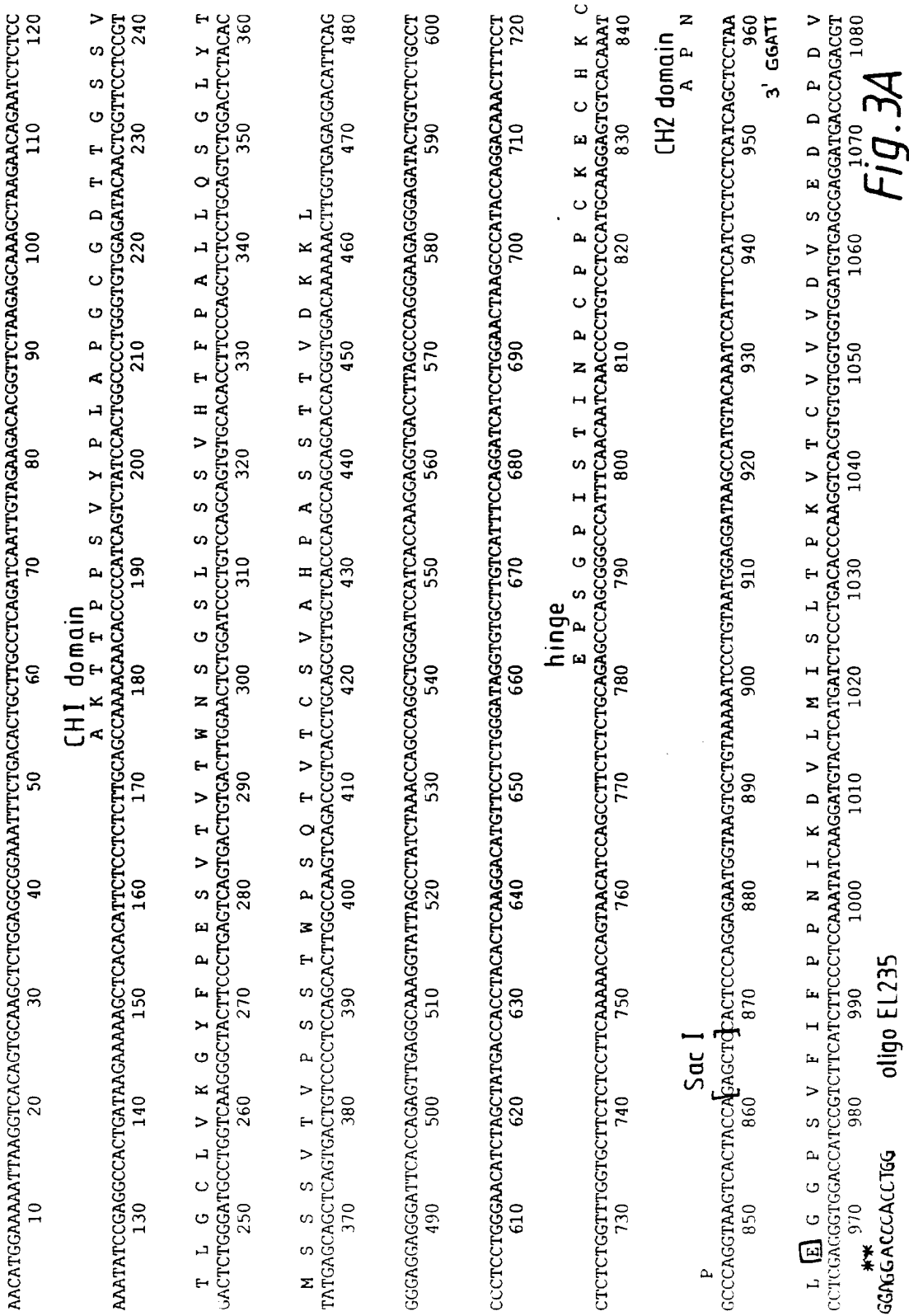


Fig. 2



Q I S W F V N N V E V H T A Q T Q T H R E D Y N S T I R V V S T L P I Q H Q D W
 CCAGATCAGCTGGTTGTGAACAACGTTGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGATTACAACAGTACTATCCGGTGGTACAGACCCCTCCCATCCAGCACCAGGACTG
 1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200

M S G K E F K C K V N N K D L P S P I E R T I S K I K
 GATGAGTGGCAAGAGTTCAATGCAAGTCAACAACAAGACCTCCCATCACCCTCGAGAGAACCATCTCAAAAATTAAAGGTGGACCTGCAGGACAACTGCATGGGGGCTGGGATG
 1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320

CH3 domain SacI (removed)
 G L V R A P Q V Y I L P P A E
 GGCAATAAGAATAAATGTCTATGTGGACAGCTTCCACTTCAGCCATGACCTCTATGTATGTCTTAACCCACAGGGCTAGTCTAGCTGACAAAGTATACATCTTGCCGCCACCAGCAG
 1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440

Q L S R K D V S L T C L V V G F N P G D I S V E W T S N G H T E E N Y K D T A P
 AGCAGTTGTCCAGGAAGATGTCAGTCTCAGTTCACCTGGTGGCTTCAACCCTGGAGACATCAGTGTGGAGTGGACCAATGGGCATACAGAGGAGAACTACAAGGACACCCGCAC
 1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560

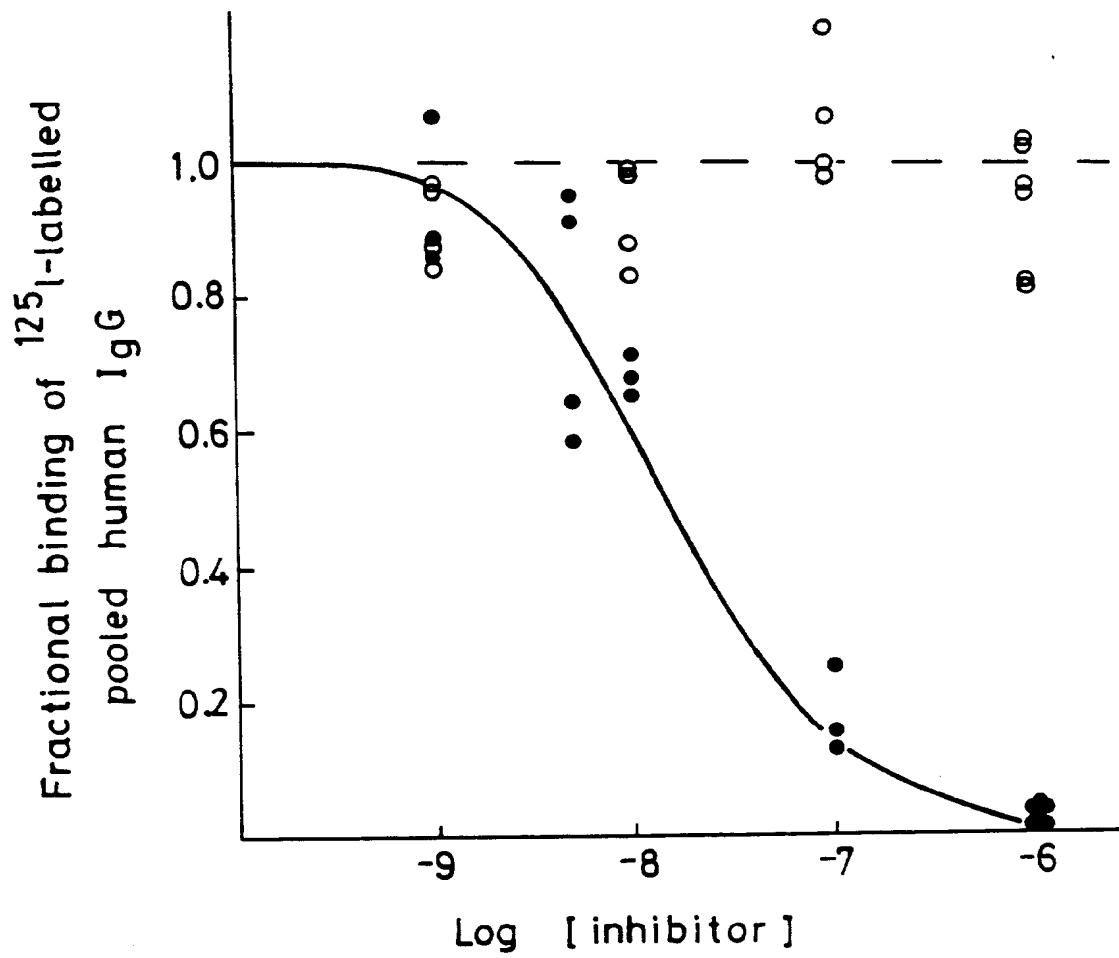
V L D S D G S Y F I Y S K L N M K T S K W E K T D S F S C N V R H E G L K N Y Y
 CAGTCCCTAGACTCTGACGGTTCTTACTTATATAGCAAGCTCAATATGAAAAACAAGCAAGTGGGAGAAAAACAGATTCTCTTCATGCAACCGTGAGACACAGAGGGTCTGAAAAATTACT
 1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680

L K K T I S R S P G K * SacI
 ACCTGAAGAAGACCATCTCCGGTCTCCGGGTAAATGAGCTGAGCACCCCAAAAGCTCTCAGGTCCCTAAGAGACACTGGCACCCCATATCCATGCATCCCTTGTTATAATAAAGCATCCAG
 1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800

CAAAGCCTGGTACCATGTAAACTGTCCCTGGTCTTTCCAAAGGTATAGAGCATAGCTCACGGGCTGATAGGTCTGGCCAGGGCCGAGAACACCTTGTCTATAGGAGAGAATGAGGTT
 1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920

TCTGCTGCAT
 1930

Fig. 3B

*Fig.4*

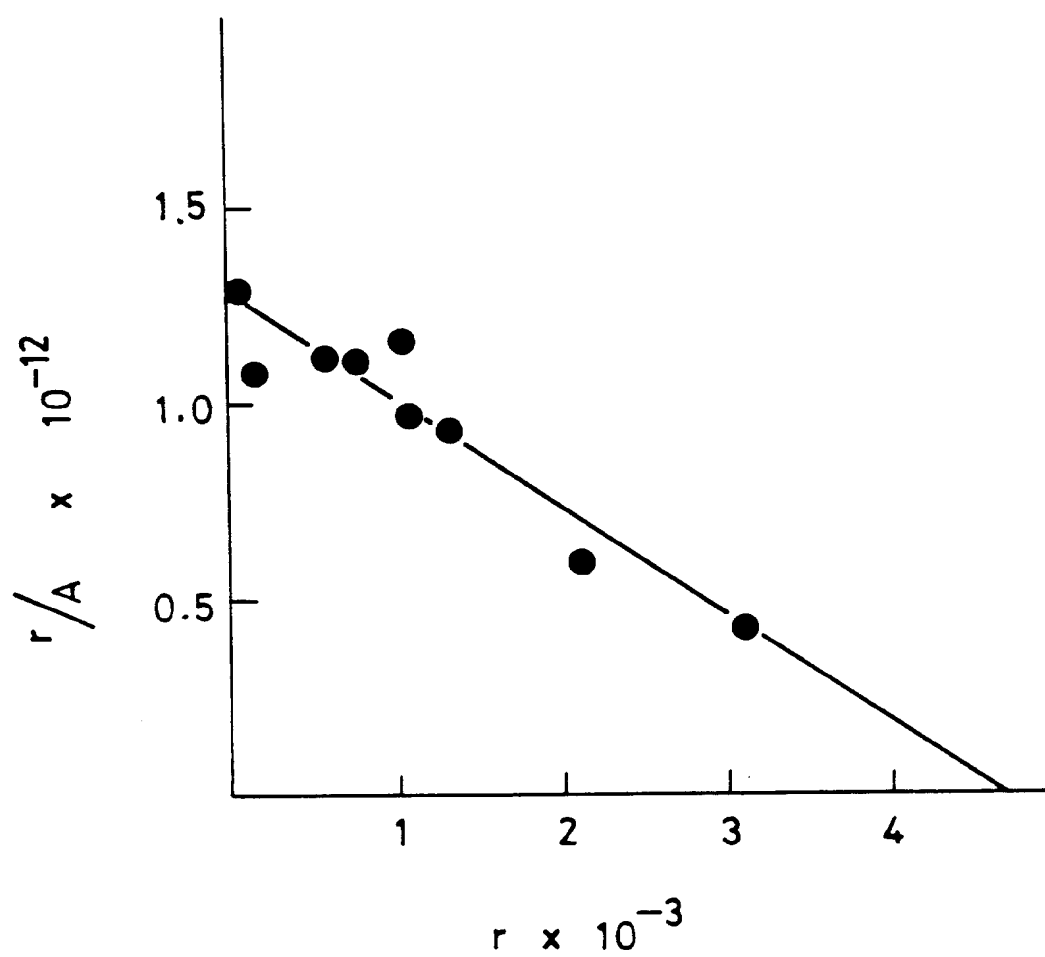
*Fig. 5*

Fig. 6

CCCTGTAATGGAGGATAAGCCATGTACAAATCCATTTCCATCTCTCCTCATCAGCTCCTA A P N

253
 L E G G P S U F I F P P N I K D U L M I
 ACCTCGAGGGTGGACCATCCGTCTTCATCTTCCCTCCAATATCAAGGATGTACTCATGA
 3' ATGAGTACC

S L T P K U T C U U U D U S E D D P D U
 TCTCCCTGACACCCAGGTCACGTGTGTGGTGGTGGATGTGAGCGAGGATGACCCAGACG
 GGAGGGACT 5' Ile253-Ala

Q I S W F U N N U E U H T A Q T Q T H R
 TCCAGATCAGCTGGTTTGTGAACAACGTGGAAGTACACACAGCTCAGACACAAACCCATA

297
 *
 E D Y N S T I R U U S T L P I Q H Q D W
 GAGAGGATTACAACAGTACTATCCGGGTGGTCAGCACCCCTCCCCATCCAGCACCAGGACT
 3' TCCTAATGCGGTCATGAT 5' Asn297-Ala

318 320 322 333
 * * *
 M S G K E F K C K U N N K D L P S P I E
 GGATGAGTGGCAAGGAGTTCAATGCAAGGTCAACAACAAGACCTCCCATCACCCATCG
 3' CACCGTTCCGGAAGT 5' Glu318-Ala 3' TGGGTAGC

3' CCTCAGCGGACGTTCC 5' Lys320-Ala
 3' TTACGCGGCAGTTG 5' Lys322-Ala

337
 *
 R T I S K I K
 AGAGAACCATCTCAAAATTAAAGGTGGGACCTGCAGGACA
 GGTCTT 5' Glu333-Ala
 3' GGTAGCGGTTTTAA 5' Ser337-Ala

Fig. 7